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Identification of the Region Responsible for Fibril Formation in the CAD Domain of Caspase-Activated DNase

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Caspase-activated DNase (CAD) has a compact domain at its N-terminus (CAD domain, 87 amino acid residues), which comprises one α-helix and five β-strands forming a single sheet. The CAD domain of CAD (CAD-CD) forms amyloid fibrils containing α-helix at low pH in the presence of salt. To obtain insights into the mechanism of amyloid fibril formation, we identified the peptide region essential for fibril formation of CAD-CD and the region responsible for the salt requirement. We searched for these regions by constructing a series of deletion and point mutants of CAD-CD. Fibril formation by these CAD-CD mutants was examined by fluorescence analysis of thioflavin T and transmission electron microscopy. C-Terminal deletion and point mutation studies revealed that an aromatic residue near the C-terminus (Trp81) is critical for fibril formation. In addition, the main chain conformation of the β5 strand, which forms a hydrophobic core with Trp81, was found to be important for the fibril formation by CAD-CD. The N-terminal 30 amino acid region containing two β-strands was not essential for fibril formation. Rather, the N-terminal region was found to be responsible for the requirement of salt for fibril formation.

Key words: amyloid fibril, CAD domain, caspase-activated DNase, charge shielding effect, proline-scanning.

Abbreviations: CAD, caspase-activated deoxyribonuclease; CAD-CD, CAD domain of caspase-activated deoxyribonuclease; ThT, thioflavin T; TEM, transmission electron microscopy.

Some proteins are known to form amyloid fibrils regardless of whether they cause serious diseases or not (*1–4*). These proteins exhibit no similarity in either their amino acid sequence or tertiary structure in the native state. In most cases, the amyloid fibrils share a common structural motif, the cross-β structure, which is composed of pairs of β-sheets in a helical arrangement (*5–7*). On the other hand, there are many non-amyloidogenic fibrous proteins with a helical structure such as α-keratin (*8*) and collagen (*9–11*), and β-structures are often found in viral fiber proteins (*12*). These facts lead to the idea that there might be various types of fibril architecture. Recently, the presence of amyloid fibrils that do not adopt the common cross-β structure but contain an α-helix has been suggested (*13*, *14*).

Caspase-activated DNase (CAD) degrades chromosomal DNA through the action of caspase in the process of apoptosis (*15*). In the N-terminal region of CAD, there is a compact domain, designated as the CAD domain, which consists of 87 amino acids. The tertiary structure of the CAD domain of CAD (CAD-CD) shows it belongs to the ubiquitin superfold family, and contains an α-helix and a β-sheet consisting of five β-strands (*16*, *17*). We previously reported that, at low pH (below 4) and in the presence of anions, CAD-CD forms amyloid fibrils without a lag time (*18*). The secondary structure of CAD-CD in the fibril state comprises not only β-sheet but also α-helix, as found in CD, FTIR, and X-ray fiber diffraction experiments. We have also shown by means of CD and fluorescence experiments that aromatic side chains have a defined orientation and are in the hydrophobic environment occurring with the fibril formation. Experiments involving a series of salts revealed that the ion shielding by anions plays an important role in the fibril formation by CAD-CD.

Many amyloidogenic proteins possess key regions that are specific to each amyloid protein. Identifying such a key region would provide a clue for understanding the mechanism of amyloid fibril formation. In this study, we identified the peptide regions which dominate the fibril formation. We reveal the essential region for the fibril formation by CAD-CD by means of deletion analysis and the importance of the hydrophobic environment of an aromatic side chain for the fibril formation. We also discuss the region responsible for the salt requirement of fibril formation by CAD-CD.

EXPERIMENTAL PROCEDURES

Protein Preparation—The coding sequence of CAD-CD (*16*) was cloned into the pET32LIC vector (Novagen, Darmstadt, Germany), and the digestion sequence of Enterokinase (DDDDK) was converted to LEVLFQGP, which is recognized by PreScission Protease (Amersham

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Biosciences, Piscataway, NJ). The resulting plasmid was introduced into *Escherichia coli* strain BL21(DE3), and the expression of CAD-CD fused with thioredoxin was induced by isopropyl-β-D-thiogalactopyranoside. Protein purification was carried out on a Ni^{2+} chelating column (Amersham Biosciences), followed by removal of the fusion tag with PreScission Protease and by reverse phase HPLC on a C_4 column (Waters, Milford, MA). The final product was identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry with a Voyager DE-STR instrument (Applied Biosystems, Foster City, CA). The protein concentration was determined from the absorbance at 280 nm (*19*).

Construction of Mutants—Deletion mutants were constructed by amplification of the deleted coding sequences by PCR and subsequent recloning into an expression plasmid. Point mutants were constructed with a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Each of the C-terminal deletion mutants is named after the position of the stop codon. For mutant H82stop, a stop codon is introduced at the position of His82 and the C-terminal end residue is Trp81. The N-terminal deletion mutants are named after the position of the N-terminus. For mutant E31start, the N-terminal sequence is GPE^{31}LLRK, the residues underlined being derived from the recognition sequence with PreScission Protease. Point mutants are named in the conventional manner. The mutant in which Trp81 is replaced by Ala is designated as W81A.

Assaying of Fibril Formation—Amyloid fibril formation was examined as the fluorescence intensity of thioflavin T (ThT) (*20*, *21*). Samples of 50 μM protein in 20 mM Gly-HCl (pH 3.4) containing various concentrations of NaCl were incubated at 35°C for 20 h. Then, each of the samples was diluted 100-fold with 50 mM Gly-NaOH (pH 8.5) containing 5 μM ThT. The amount of amyloid fibrils was estimated from the fluorescence intensity of the solution (Ex, 455 nm; Em, 490 nm) at 6°C using an RF-5300PC spectrofluorophotometer (Shimadzu, Kyoto, Japan). The formation of amyloid fibrils was also confirmed by transmission electron microscopy (TEM) with a TecnaiG^2F20 transmission electron microscope (FEI, Eindhoven, Netherlands) operated at an accelerating voltage of 100 kV. Negative staining was performed according to the conventional method as described elsewhere (*22*). Congo red staining was performed as follows (*23*). Amyloid fibrils were dialyzed in water and air-dried on a glass slide. Then, each sample was stained with a solution comprising 80% ethanol, a saturated concentration of NaCl, and a saturated concentration of Congo red. The sample was air-dried and then examined under a light microscope using a crossed polarizer.

RESULTS

C-Terminal Deletion Analysis—We performed deletion analysis to identify the key peptide region for the fibril formation by CAD-CD. In order to evaluate the importance of the C-terminal region of CAD-CD for its fibril formation, we constructed C-terminal deletion mutants and examined their fibril formation. Deletion of 10 residues from the C-terminus prevented the fibril formation, despite the fact that all secondary structural elements remained intact (Fig. 1). We performed further analyses with nine additional deletion mutants through ThT fluorescence experiments after incubation with 0, 50, and 100 mM salt at 35°C for 20 h (Fig. 2A). Incubation for 20 h should have been sufficient to assess the fibril formation by CAD-CD (*18*). Deletion of up to six residues did not affect the fibril formation by CAD-CD. However, fibril formation did not occur for CAD-CD lacking more than seven residues from the C-terminus, Trp81 having been deleted (Fig. 2A). The fibril formation was suppressed to about 20% for the H82stop

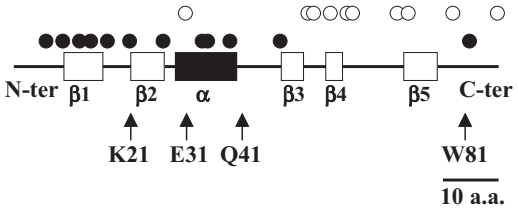

Fig. 1. **Schematic drawing of the sequence of CAD-CD**. The black box and white boxes represent the α-helix and β-strands, respectively. Closed circles and open circles indicate the positions of positively charged residues (Lys, Arg, and His) and negatively charged residues (Glu and Asp), respectively. The positions of Lys21, Glu31, Gln41, and Trp81 are indicated by arrows.

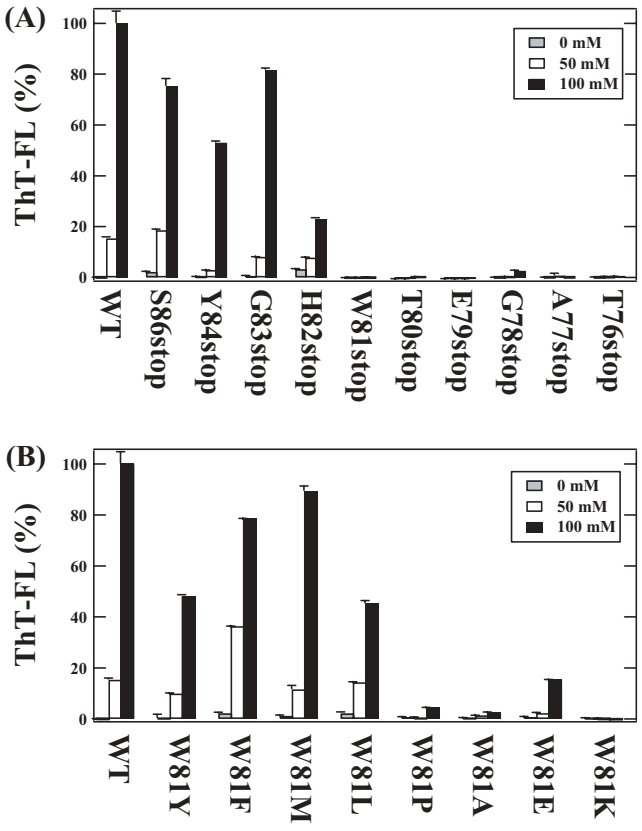

Fig. 2. **NaCl-dependent fibril formation by the mutants**. CAD-CD variants (50 μM) were incubated at 35°C for 20 h in 20 mM Gly-HCl (pH 3.4) containing 0, 50, or 100 mM NaCl. The fluorescence of a ThT solution was expressed as a percentage of that with the wild-type incubated with 100 mM NaCl. Panels A and B show C-terminal deletion mutants and point mutants as to Trp81, respectively.

mutant (Fig. 2A). We correlate this suppression with the created C-terminal end that affects the hydrophobicity of the C-terminal Trp81 (described later). These results indicate that the C-terminal six residue region is not essential, but that the peptide region containing Trp81 plays an indispensable role in the fibril formation.

Point Mutation Analysis at Trp81—In the deletion studies described above, the C-terminal region containing Trp81 was found to be critical for the fibril formation by CAD-CD. We carried out point mutation analysis at Trp81 and examined the role of the side chain at this position (Fig. 2B). We correlate the tendency to form amyloid fibrils with the volume and hydrophobicity of the side chain of the introduced amino acid. When Trp81 was substituted by a bulky hydrophobic residue (Tyr, Phe, Met, or Leu), the mutant CAD-CD formed amyloid fibrils like the wild-type. Fibril formation was suppressed for mutants W81P and W81A. A little (approximately 20%) fibril formation was observed for mutant W81E, in which the side chain of the introduced Glu has no charge at the pH used in this experiment (pH 3.4). Replacement by a positively charged residue (Lys) completely prevented the formation of amyloid fibrils. These results lead to the conclusion that the hydrophobic environment of Trp81 is required for formation of amyloid fibrils by CAD-CD.

Proline-Scanning around the β*5-Strand*—Our previous study showed that Trp81 is in a more hydrophobic environment on fibril formation, as revealed by fluorescence measurements (*18*). The side chain of Trp81 forms a local hydrophobic core with neighboring residues in the primary structure (*16*). Therefore, the conformation of the region upstream of Trp81 may be critical for the fibril formation. In order to confirm this, we examined the role of this region by locking the main chain conformation by sequentially introducing a Pro residue at each position.

The fibril formation by the proline-scanned mutants is shown in Fig. 3. The mutation sites spanned from Glu79 to Glu71, which corresponds to the β5 strand (Fig. 1, Ala70–Leu75) in the native state, and the sites between the β5 strand and Trp81. Replacement of Glu79 and Gly78 by Pro did not affect the fibril formation. When Pro was introduced at the next five positions, the fibril formation was suppressed. In particular, substitution of Leu75, Leu74, and Leu73 by Pro completely prevented fibril formation. As the mutation site moved toward the N-terminal, the mutants started to form amyloid fibrils, as observed for L72P and E71P (Fig. 3A). Mutant T80P could not be obtained because it did not accumulate in the *E. coli* cells for an unknown reason. The amount of the amyloid fibrils, as estimated from the fluorescence intensity of ThT, did not change on further incubation at 35°C for one week (data not shown).

TEM images of representative fibrils are presented in Fig. 3B. A curvilinear shape was observed for the fibrils of the other proline-scanned mutants as well as for those of the C-terminal-deleted and point mutants (data not shown). These amyloid fibrils exhibited apple-green birefringence under cross-polarized light when stained with Congo red dye (Fig. 3C), indicating that they have a tinctorial property characteristic of amyloid fibrils.

The results of proline-scanning mutation suggest that the regular secondary structure around the C-terminal

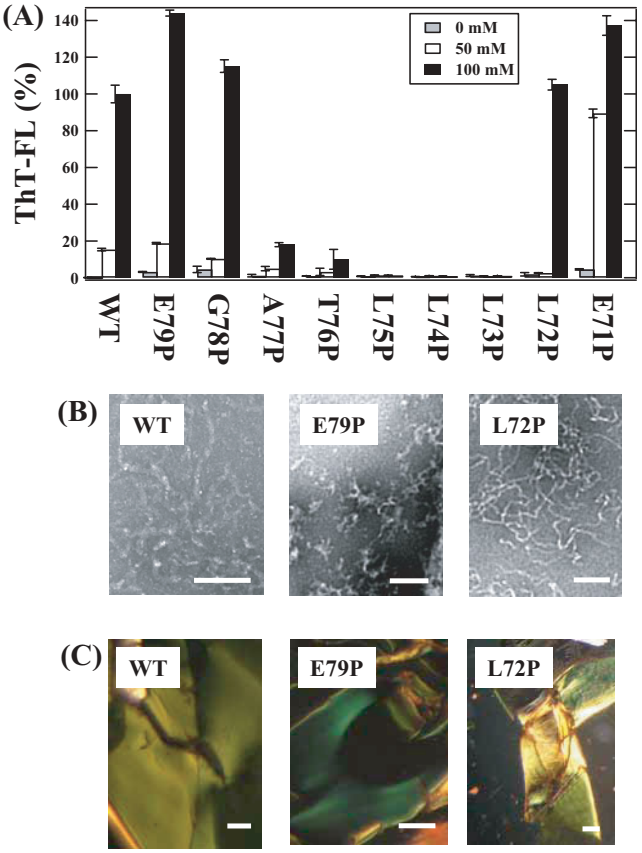

Fig. 3. **Fibril formation by the proline-scanned mutants**. (A) Experiments were carried out as described in the legend to Fig. 2. (B) TEM images of representative fibrils formed by the wild-type, E79P, and L72P are shown. Scale bars represent 100 nm. (C) Congo red staining of the same fibrils as shown in panel B. Scale bars represent 100 μm.

part of the β5 strand, which spans from Leu73 to Ala77, is critical for the fibril formation by CAD-CD.

Role of the N-Terminal Region—Next, we performed N-terminal deletion studies in order to evaluate the role of the N-Terminal region. N-terminal deletion mutants were obtained by removing either the β1 strand, the β1 and β2 strands, or these two β-strands with the α-helix, the mutants being named K21start, E31start, and Q41start, respectively (Fig. 1). Fibril formation by the wild-type CAD-CD is dependent on the presence of salt. Interestingly, however, K21start and E31start formed amyloid fibrils in the absence of salt (Fig. 4). This means that deletion of up to 30 residues at the N-terminus abolished the salt requirement and increased the tendency of amyloid fibril formation by CAD-CD. Almost all the mutants were soluble and partially denatured under the examined buffer conditions (pH 3.4). Mutant Q41start was insoluble at pH 3.4 but soluble at pH 2.4. The insoluble Q41start at pH 3.4 did not comprise amyloid fibrils as judged from the ThT fluorescence. Q41start did not form amyloid fibrils at pH 2.4 (Fig. 4), at which the wild-type did (data not shown). This suggests that deletion of the N-terminal 40 residues to remove the α-helix reduced the ability of CAD-CD to form amyloid fibrils. The results of the N-terminal deletion study indicated that at least

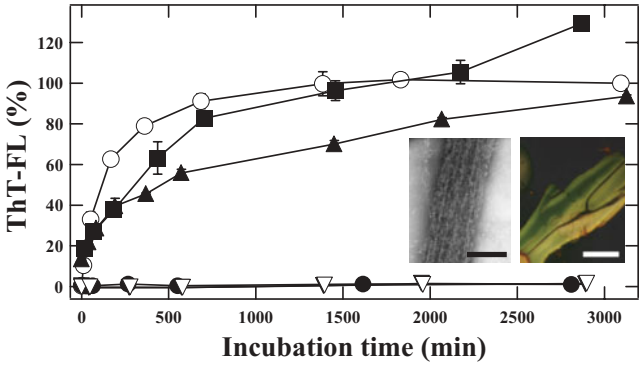

Fig. 4. **Time course of fibril formation by the N-terminal deletion mutants**. The fibril content was expressed as the fluorescence of a ThT solution and plotted versus incubation time at 35°C. The control value (100%) is the fluorescence intensity with the wild-type incubated with 100 mM NaCl at the saturated phase. Since Q41start was not soluble at pH 3.4, 10 mM HCl was used instead of 20 mM Gly-HCl buffer (pH 3.4) for this mutant. Insets show a TEM image (left) and Congo red staining (right) of an E31start fibril. Scale bars represent 500 nm (left) and 200 μm (right). Open circles, wild-type with 100 mM NaCl; solid circles, wild-type without NaCl; solid triangles, K21start without NaCl; solid squares, E31start without NaCl; inverted open triangles, Q41start with 100 mM NaCl; inverted solid triangles, Q41start without NaCl.

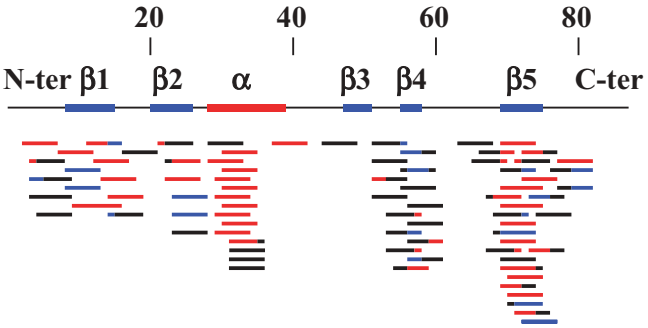

Fig. 5. **Secondary structures preferred by partial sequences of CAD-CD**. The secondary structures of proteins containing five-amino-acid-long partial sequences of CAD-CD are illustrated. The top bar represents the distribution of the secondary structural elements in CAD-CD, and the bottom bars represent the results of the database search, which was performed with the Parallel Protein Information Analysis (PAPIA) system (*28*). Red, blue, and black bars indicate α-helices, β-strands, and other structures, respectively. The secondary structures were taken from the following PDB codes: 1B65, 1GOS, 7FAB, 1MCW, 1JAT, 1C5A, 1RGS, 1DBY, 1K94, 1JUO, 1REQ, 1EJD, 1L5A, 1ES9, 1FXW, 1K1A, 2DNJ, 1D6R, 1K20, 1H32, 1M32, 1FXZ, 1HTY, 1LIU, 1RMI, 1LE6, 1QMB, 1JPX, 1D0N, 1INP, 1CEO, 1M0S, 1L8A, 1IRU, 1T7P, 1HQ3, 1VID, 1J99, 1N1B, 1GVE, 1GH7, 1AYZ, 3NLA, 1FGJ, 1KB9, 2MYS, 1QKS, 1M8Q, 1K1C, 8ABP, 1EB6, 1FYC, 1QRE, 1A6Q, 1A1S, 1A4E, 1QUS, 1JMU, 1PVD, 1NSA, 1JJV, 2PHL, 1B41, 1BI7, 1IEG, 1B16, 1HO1, 1D9S, 1A5E, 1JSW, 1GNG, 1WPO, 1JQJ, 1PSD, 1FYE, 1CJY, 1JI7, 1G8P, 1ADO, 1KIT, 1H2W, 1DP4, 1TYF, 1BJW, 1E43, 1GAQ, and 1H2W.

30 amino acids at the N-terminus are not essential for the fibril formation by CAD-CD but are responsible for the requirement of salt for fibril formation.

DISCUSSION

Because the characteristics of wild-type fibrils were also found for the fibrils formed by the mutant proteins examined in this study, mutation analysis is a good method for elucidating the mechanism underlying the fibril formation by CAD-CD. Fibril formation by the mutant proteins proceeded without a lag time, and was almost complete after incubation for 20 h. The mutant proteins showed similar spectral properties to those of the wild-type along with the fibril formation (data not shown). These observations indicate that the fibril formation by the mutant proteins occurred in the same manner as for the wild-type.

The essential region for fibril formation was shown to be at least 40 amino acids long, because fibril formation was not observed for Q41start or W81stop. This is a relatively long peptide region, because several shorter peptides have been shown to be sufficient for the formation of amyloid fibrils. For example, Hasegawa *et al.* (*24*) reported that an 11-amino-acid-long partial peptide of β_2-microglobulin spontaneously forms amyloid fibrils. As our C-terminal deletion study revealed the strict necessity of Trp81 (Fig. 2A), we further examined the importance of the hydrophobicity at this position by means of site-directed mutagenesis and showed the indispensable role of the C-terminal hydrophobic core in the fibril formation by CAD-CD (Fig. 2B). Native state CAD-CD contains a hydrophobic core near its C-terminus, in which Trp81 is present (*16*). The main chain conformation around the β5 strand, which belongs to the hydrophobic core with Trp81, was critical for the fibril formation, as revealed on proline-scanning (Fig. 3A). Therefore, it is likely that the structural change affecting the hydrophobic core is a key factor for the formation of amyloid fibrils. This conclusion is consistent with the results of our previous spectroscopic study (near UV CD and fluorescence measurements), which demonstrated that the environment around Trp81 significantly changes upon fibril formation (*18*).

In the course of our deletion study, we found that the N-terminal region acts as an anion sensor regulating the fibril formation. The fibril formation by the wild-type CAD-CD is promoted by the charge shielding effect of anions (*18*). Among the mutants employed in this study, only the N-terminal deletion mutants formed amyloid fibrils without salt (Fig. 4). Therefore, this region seems to be responsible for the interaction with anions and thus for the salt requirement for fibril formation. In the sequence of CAD-CD, the charge distribution is clearly distinctive. The N-terminal half is positively charged while the C-terminal half is negatively charged (Fig. 1). These facts indicate that the site of interaction with anions exists in the N-terminal region. It is likely that in the absence of anions the N-terminal region prevents the fibril formation due to its positive charge and that the charge shielding effect of anions cancels out the inhibitory effect of the N-terminal region. In addition, mutant E31start forms thick and straight fibrils (Fig. 4). At present, factors that determine the morphology of CAD-CD fibrils are unclear.

It is noteworthy that amyloid fibrils formed by CAD-CD contain a helical structure, as judged from the results of CD, Fourier transform infrared, and X-ray fiber diffraction studies (*18*). The helical part of CAD-CD may be contributed from the N-terminal region, where the molten globule state is stabilized through interaction with anions. Another possibility for the helical region of CAD-CD fibrils

was revealed by a database search. Although CAD-CD is rich in β-strands, it contains many sequences that tend to adopt an α-helix in other proteins. Such sequences frequently appear around the β5 strand (Fig. 5), and they adopt a helical structure in other proteins such as L-aspartate ammonia-lyase (*25*), D-3-phosphoglycerate dehydrogenase (*26*), the 38 kDa subunit of magnesium chelatase (*27*), and so forth. Interestingly, this region is critical for fibril formation, as revealed on proline-scanning mutagenesis (Fig. 3). Thus, it is possible that the conformation around the β5 strand changes to α-helix during fibril formation and that replacement with proline residues prevented fibril formation by locking the main chain conformation around the β5 strand. The obvious description regarding the conformational change upon fibril formation will be obtained through structural studies on amyloid fibrils formed by CAD-CD.

Identifying a region essential for fibril formation provides a clue for understanding the mechanism by which they are formed. Such a key region was identified for CAD-CD in this study. Moreover, the region responsible for the effect of anions during fibril formation was also determined. This work provides new insights into the structural basis of the fibril formation by proteins.

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